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ANION TRANSPORT IN SINGLE ERYTHROCYTE GHOSTS MEASURED BY FLUORESCENCE MICROPHOTOLYSIS

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Fluorescence microphotolysis was used to measure in single resealed human erythrocyte ghosts the band 3-mediated transport of the fluorescent anion *N*-(7-nitrobenzofurazan-4-yl)-taurine (NBD-taurine). Transport was reduced to less than 5% of the control by the specific inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS). The accuracy of the determination of the rate constant for NBD-taurine influx was approximately ±15% as calculated from repetitive measurements in individual ghosts. The sample population distribution of the rate constant was slightly skewed towards values larger than the mean. The rate of NBD-taurine transport showed an optimum near pH 7. The Arrhenius plot was linear in the range from 28.5°C to 51°C with an apparent activation enthalpy of 21.4 kcal/mol.

Anion transport across the erythrocyte membrane is mediated by the band 3 protein. Both anion transport and the band 3 protein were the object of many physical and chemical studies. The wealth of information obtained has led to rather detailed suggestions about the nature of anion transport (for reviews, see Refs. 1–4). However, most previous studies were performed with cell populations and hence provided information only on average properties of the cells. The distribution of properties amongst the individual cells of the population remained unresolved. Very recently single-cell flux measurement has become feasible [5–7].

Fluorescence microphotolysis (Ref. 8, for review, see Ref. 9) has been employed for flux measurement in single erythrocyte ghosts [5,6], primary hepatocytes [7], isolated liver cell nuclei [5] and nuclear ghosts [10]. The principle of the method consists of equilibrating cells with a fluorescent solute, measuring intracellular fluorescence in a single cell by microfluorometry, rendering the fluorescent molecules in the intracellular space of that cell non-fluorescent by a high-intensity laser pulse, and following on the influx into the cell of unbleached fluorescent molecules from the medium by microfluorometry. The fluorescent anion *N*-(7-nitrobenzofurazan-4-yl)taurine (NBD-taurine) was introduced by Eidelman and Cabantchik to study band 3-mediated anion transport in intact erythrocytes by fluorometry of cell suspension [11–13]. In the present report measurements of the influx of NBD-taurine into single erythrocyte ghosts are presented. Some basic features of both the method (accuracy, absence of photochemical artefacts) and the transport system (pH and temperature dependence) are described. For the first time the distribution of the influx rate constant k amongst individual cells of a population could be determined.

Resealed erythrocyte ghosts were prepared as described previously [14] using human blood (O, Rh^+) from a local blood bank. The final medium

Abbreviations: NBD, *N*-(7-nitrobenzofurazan-4-yl); DIDS 4,4′-diisothiocyanostilbene-2,2′-disulfonate.

contained 20 mM ethylene diaminotetraacetate (EDTA) and 130 mM sodium chloride, pH 7.4. NBD-taurine (Molecular probes, Junction City, U.S.A.) was added to the ghost suspension at a final concentration of 1 mM. Subsequently, the suspension was adjusted to the desired pH and incubated at 37°C until equilibrium was established. In contrast to the procedure employed previously [5,6] the ghost suspension was not placed as a very thin layer between a glass slide and a cover slip but was filled into a flat glass capillary (a 'microslide' of 50 μm pathlength, Camlab, Cambridge, U.K.). This proved to be an extremely simple method beneficial for the integrity of the ghosts. The microslide was mounted on a small device designed for temperature control. The device consisted of a brass plate with a central hole of 5 mm diameter for illumination of the specimen on the microscope stage and with internal channels for perfusion from the thermostat. The device was turned upside down and kept in this position for a few minutes. It was then placed right-side up on the stage of the microscope and the microscope was focussed on ghosts adhering to the upper glass-medium interface using 100-fold, n.a. 1.3, oil immersion, phase contrast objective lens. The objective lens was also temperature-controlled by means of a metal ring which was perfused parallel to the plate carrying the capillary. The temperature of the specimen in its final position was measured by microprobe thermometer (Bailey Instruments, Saddle Brook, U.S.A., model BAT-12) with an accuracy of approximately ± 0.2°C.

Flux measurements were performed essentially as described [5,6]. The 4765 nm line of an argon laser was used to illuminate and bleach homogeneously a circular area of 2.0 μm radius in the interior of a red cell ghost that was located in the focal plane. Fluorescence originating from the illuminated area was measured by single-photon counting equipment. On its way to the photomultiplier fluorescence passed through a photometric attachment in which the measured area was selected by means of a diaphragm located in an image plane. The apparent radius of the diaphragm was somewhat smaller than that of the illuminated area. The intensity and duration of the photolyzing laser pulse were adjusted such that the fluorescence of ghosts was reduced to approx. 30%. With higher intensities ghost fluorescence could be reduced to less than 20%. If the same pulse was delivered to a ghost-free location of the medium no effect at all on the measuring signal was observed. These control experiments show that in the employed set-up fluorescence was recorded only from a layer of the suspension not much thicker than that of the ghosts, i.e. 2–3 μm. Out-of-focus fluorescence was almost completely rejected by optical means (high aperture of the objective lens, small diaphragm in the photometric attachment). Furthermore, diffusion of NBD-taurine in the medium was fast enough to establish diffusional equilibrium after photolysis within a fraction of the sampling time of 0.5 s.

The recovery of the intracellular fluorescence after photolysis by influx of extracellular unbleached NBD-taurine into the cell was analyzed for a mobile and an immobile fraction of fluorophores [6]. However, with NBD-taurine no indication of an immobile fraction was observed, i.e. $F(\infty)$, the intracellular fluorescence measured a long time after photolysis, always returned to the level $F(-)$ observed before photolysis. Therefore, the rate constant k of influx can be obtained by fitting the experimental data to the equation:

$$\ln((F(-)-F(0))/(F(-)-F(t))) = kt \qquad (1)$$

where $F(0)$ is fluorescence immediately after photolysis and t is time.

A representative example of a flux measurement is given in Fig. 1. The raw data are plotted in Fig. 1A. The three steps of the experiment, measurement of $F(-)$, reduction to $F(0)$ by photolysis and measurement of $F(t)$, can be clearly recognized. The sensitivity of NBD-taurine transport to the action of a specific inhibitor of anion transport is evident by comparison of curves a and b. Curve a was obtained with an untreated ghost whereas curve b relates to a ghost preincubated for 30 min at 37°C with 50 μM of the band 3-specific inhibitor 4,4′-diisothiocyanostilbene-2,2-disulfonic acid (DIDS). An example for the evaluation of the data is shown in Fig. 2B where the data of Fig. 1A have been replotted according to Eqn. 1 and fitted by linear regression employing appropriate weighing factors. In the example the rate constant k amounted to 0.0237 s^{-1} for the untreated and to

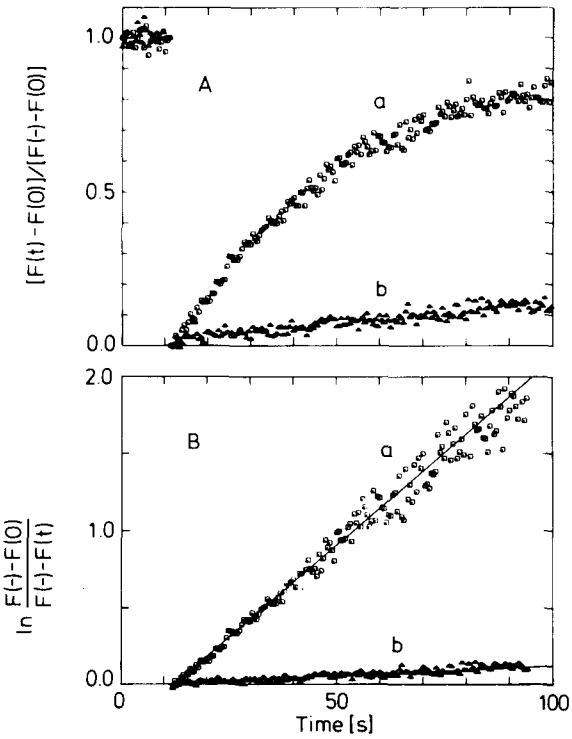

Fig. 1. Representative examples of single-cell flux measurement by fluorescence microphotolysis. Resealed erythrocyte ghosts were prepared in 20 mM EDTA/130 mM NaCl (pH 7.4), equilibrated with 1 mM NBD-taurine and measured at 47.5°C. (A) Raw data in normalized form where $F(-)$, $F(0)$ and $F(t)$ represent, respectively, intracellular fluorescence before, immediately after, and at time t after photolysis. The actual degree of photolysis was $F(0)/F(-) = 0.3$. (a) untreated ghosts and (b) ghosts preincubated in 50 μM of the band 3-specific inhibitor DIDS. (B) Semilog representation of same data as in panel A (Eqn. 1).

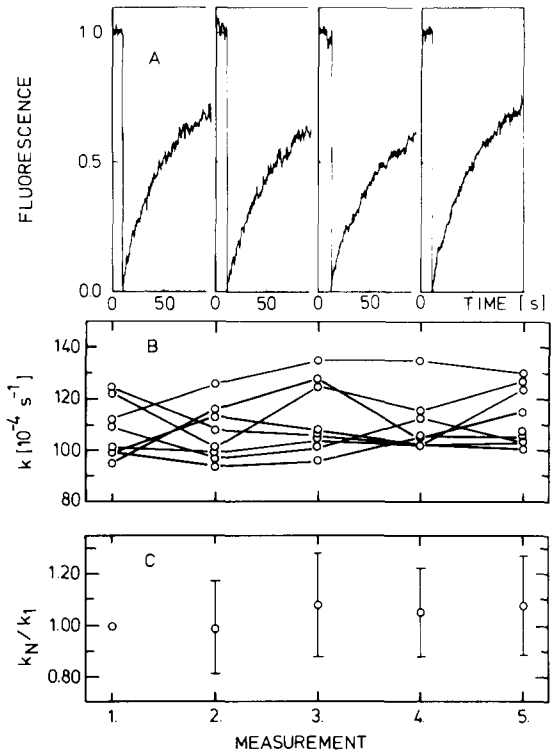

Fig. 2. Repetitive measurements on individual ghosts (45.0°C, pH 7.4). (A) Example of repetitive measurements on an individual ghost. The ordinate gives normalized fluorescence as in Fig. 1A. Each measurement extended over a period of 100 s. Between measurements the ghost was left in the dark until fluorescence had recovered to the level before photolysis. (B) The absolute rate constant k of eight ghosts each subjected to five consecutive measurements. The values of individual ghosts are connected by straight lines. (C) For each ghost the rate constants were normalized to the first measurement and then averaged. The experimental uncertainty is approx. $\pm 15\%$. Within this uncertainty no systematic effect of repetitive measurement on the rate constant is apparent. Note that zero on the ordinate is suppressed.

0.0015 s^{-1} for the DIDS-treated ghosts (47.5°C, pH 7.4). In general NBD-taurine transport was inhibited by more than 95% in the presence of 50 μM DIDS.

Repetitive measurements on eight individual ghosts are summarized in Fig. 2. At 45.0°C and pH 7.4 each ghost was measured five times allowing for complete equilibration between measurements. Panel A displays the measurements of one particular ghost (measurements 1–4 are shown, the fifth is omitted). Panel B gives the rat constants of each ghost. In panel C rate constant of the second to fifth measurement were normalized to the first measurement for each ghost individually and then averaged. Within an experimental accuracy of approx. $\pm 15\%$ no systematic effect of repetitive photolysis on the rate constant is apparent.

The distribution of the rate constant k in a sample of 120 ghosts is shown in Fig. 3. Based on a Gaussian distribution the mean $\pm$ S.D. of k was 0.0074 ± 0.0023 s^{-1} (40.0°C, pH 7.4). As stated above the reproducibility of repetitive measurements was no more than approximately 2-fold better, and hence amounted to about $\pm 15\%$. This scatter of the method tends to obscure details of

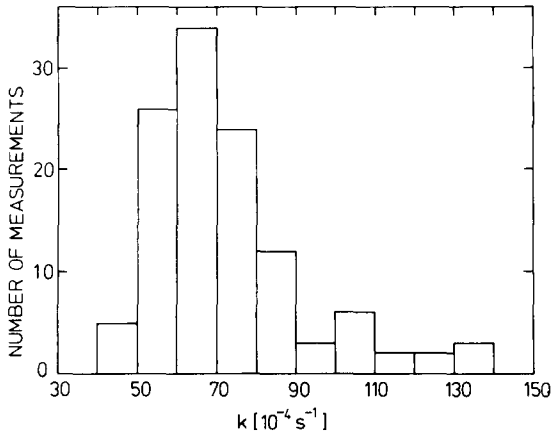

Fig. 3. Distribution of rate constant k in a sample of 120 ghosts (40.0°C, pH 7.4).

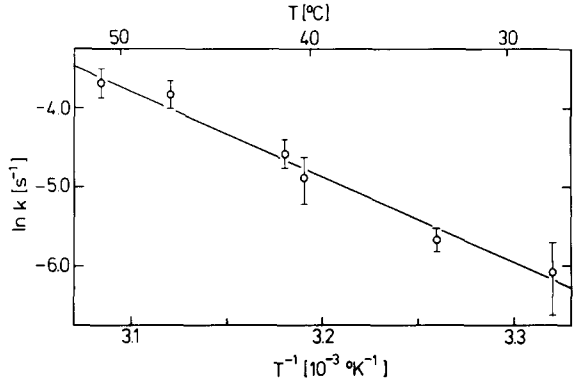

Fig. 5. Temperature dependence of the rate constant. The natural logarithm of the rate constant was plotted versus reciprocal absolute temperature. The apparent activation enthalpy is 21.4 kcal·mol^{-1}. Each value is the mean ± S.D. of 10–15 measurements.

the sample population distribution. Nevertheless the shift towards k values larger than the mean represented in Fig. 3 seems to be real. The rate constant is related to both membrane transport and cellular geometry by the equation $k = (O/V)P$ where P, V and O are permeability, volume and surface area, respectively. Therefore the frequency distribution of k may reflect the distribution of one or any combination of these parameters. The ambiguity could only be resolved by measuring cell size and membrane transport in the same individual cells.

The pH dependence of k is shown in Fig. 4 (47.0°C). A maximum is observed at about pH 7

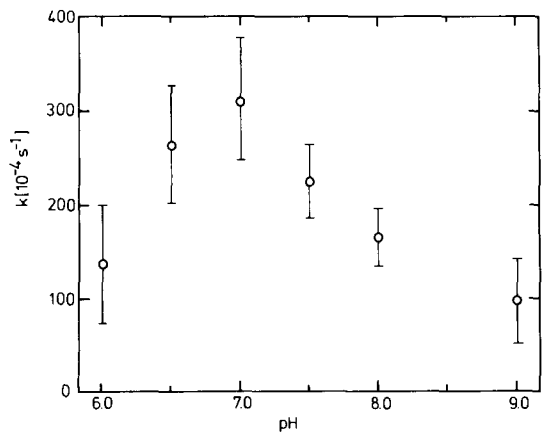

Fig. 4. pH dependence of the rate constant k. Each value represents mean ± S.D. of 10–15 measurements.

which closely agrees with measurements of NBD-taurine efflux [13] and is similar to measurements of sulfate transport [15].

The temperature dependence of k is given in Fig. 5. Over the range of 28.5°C to 51.0°C the Arrhenius plot is linear with an apparent activation energy of 21.4 kcal · mol^{-1}. A similar value of 23 kcal · mol^{-1} has been reported [13] for NBD-taurine efflux from cell populations.

In summary, the study shows that band 3-mediated anion transport can be measured in single erythrocyte ghosts. The present results are essentially similar to those obtained previously by conventional techniques that involved the sampling of large cell population. The experimental accuracy, although moderate at present, was sufficient to reveal some details of the sample population distribution. This offers new possibilities to membrane transport research. Fluorescence microphotolysis may prove particularly useful where small and/or heterogeneous samples prevail as for instance in clinical research and diagnostics.

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try, an interesting approach complementary to our method. Support by the Deutsche Forschungsgemeinschaft (PE 138/8) is gratefully acknowledged.

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